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Simple Serological Assays for Detecting Rice Tungro Viruses

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An attempt was made to produce sensitive and specific polyclonal antisera against the viruses causing rice tungro disease, and to assess their potential for use in simple diagnostic tests. Using a multiple, sequential injection procedure, seven batches of polyclonal antisera against rice tungro bacilliform virus (RTBV) and rice tungro spherical virus (RTSV) were produced. These were characterized for their sensitivity and specificity using ring-interface precipitin test and double antibody sandwich (DAS) ELISA. Thirty-one weeks after the first immunization, antiserum batch B6b for RTBV showed the highest ring interface titer (DEP = 1:1920). For RTSV, batches S3, S4b and S5b all had similar titres (DEP = 1:640). In DAS-ELISA, however, significant differences among purified antisera (IgG) batches were observed only at IgG dilution of 10^{-3}. At that dilution, IgGB4b showed the greatest sensitivity, while IgGS3 showed greatest sensitivity for RTSV. When all IgG batches were tested against 11 tungro field isolates (dual RTBV-RTSV infections) at sample dilution of 1:10, IgGB4b and IgGB6b for RTBV and IgGS3 and IgGS6b for RTSV performed equally well. However, after cross adsorption with healthy plant extracts in a specially prepared healthy plant-Sepharose affinity column, only IgGB6b could be used specifically to detect RTBV in a simple tissue-print assay.

Keywords: *Rice tungro bacilliform virus (RTBV), rice tungro spherical virus (RTSV), polyclonal antisera, protein-based tools, ELISA, cross-adsorption, diagnostic kit*

INTRODUCTION

Rice, *Oryza sativa* L., is one of the world's most important cereal crops, with an annual production of 562 million tonnes from an area of 152 million ha. More than 90% of the world's rice is produced and consumed in Asia (Hossain & Pingali, 1998). Of all the viral

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diseases on rice, tungro is the most economically devastating in the Asian tropics and it is estimated to cause annual losses of about US$1.5×10^9 (Hibino, 1996; Herdt, 1991). Tungro disease is caused by a combination of an RNA virus, rice tungro spherical virus (RTSV) and a DNA virus, rice tungro bacilliform virus (RTBV). Together in a plant, these produce the tungro disease symptoms, which include stunting, yellow or yellow-orange leaf discoloration, and reduced tillering. Plants infected with RTBV alone show similar but milder symptoms, while those infected with RTSV alone show no prominent symptoms except very mild stunting (Sta. Cruz *et al.*, 1993). After feeding on RTBV- and RTSV-infected plants, *Nephotettix virescens*, the green leafhopper vector, transmits either or both viruses. The leafhopper can acquire and transmit RTSV alone but is unable to acquire RTBV alone. Only leafhoppers that have fed previously on RTSV-infected plants are capable of acquiring RTBV from plants infected with RTBV alone (Hibino *et al.*, 1978; Hibino, 1983).

Except in advanced laboratories, tungro-infected rice plants are generally identified by visual symptoms or occasionally by insect transmission of the viruses to assay plants. However, diagnosis of the disease by symptoms alone is not reliable since other virus and non-pathogenic disorders such as nutritional deficiencies, excess water after drought, or insect injury can cause similar symptoms. Insect transmission assays are not necessarily specific for tungro, are laborious and time consuming, and require the use of virus free vector populations (Omura *et al.*, 1983). Serological techniques to detect viruses are relatively more specific, sensitive and reliable (Hibino & Kimura, 1982; Luisoni *et al.*, 1983). As long ago as the mid-1980s, Bajet *et al.* (1985) using DAS-ELISA were able to detect RTBV and RTSV separately propagated in the greenhouse. Later, this technique was used to screen breeding lines for apparent resistance or tolerance to the viruses, and to survey or monitor tungro spread through the Philippines (Flores *et al.*, 1990; Tiongco *et al.*, 1993). Despite this exposure, use of ELISA for tungro viruses is still very limited in rice-growing countries. This is because the facilities (buffers, ELISA plates, plate reader etc.), reliable antisera, and technical know-how are only available at very few locations.

Kits comprising all the components required to perform the reliable diagnosis of many virus diseases, including RTBV and RTSV (e.g. Adgen, Scotland) are being produced and marketed (Sward & Eegling, 1995). However, these are generally too expensive for resource-poor national programmes and extension services, and still require some expertise and often sophisticated equipment, such as an ELISA plate reader, to make a reliable interpretation of the results. To facilitate the national and regional research on the epidemiology, management and breeding for disease resistance to tungro it was considered important to develop specific, high affinity antisera that could be used in simple diagnostic tests at many locations in the tungro-prone regions of the world.

Polyclonal antibodies (pAbs) raised in animals that are repeatedly boosted with the same antigen often have improved properties over those produced with only three or four antigen injections. When an animal is given repeated injections with the same antigen, often a much faster, more potent, and more persistent response is induced. Serum from a primary injection only, contains a substantial proportion of IgM, whereas sera from secondary or repeatedly injected animals contain mostly IgG antibodies. The average affinity and specificity of antibodies for an antigen also increases with repeated injections. These changes are reported to continue through multiple rounds of immunization (Harlow & Lane, 1988).

The objectives of this study were to attempt to produce high titre polyclonal antisera against RTBV and RTSV, to evaluate their sensitivity and specificity towards each virus, and to assess their potential for use in simple rapid diagnostic tests.

MATERIALS AND METHODS

Antisera Production

The standard IRRI (Philippine) strains of RTBV and RTSV used in most of this work were maintained separately in rice plants, variety 'Taichung Native 1' (TN1), in the virology

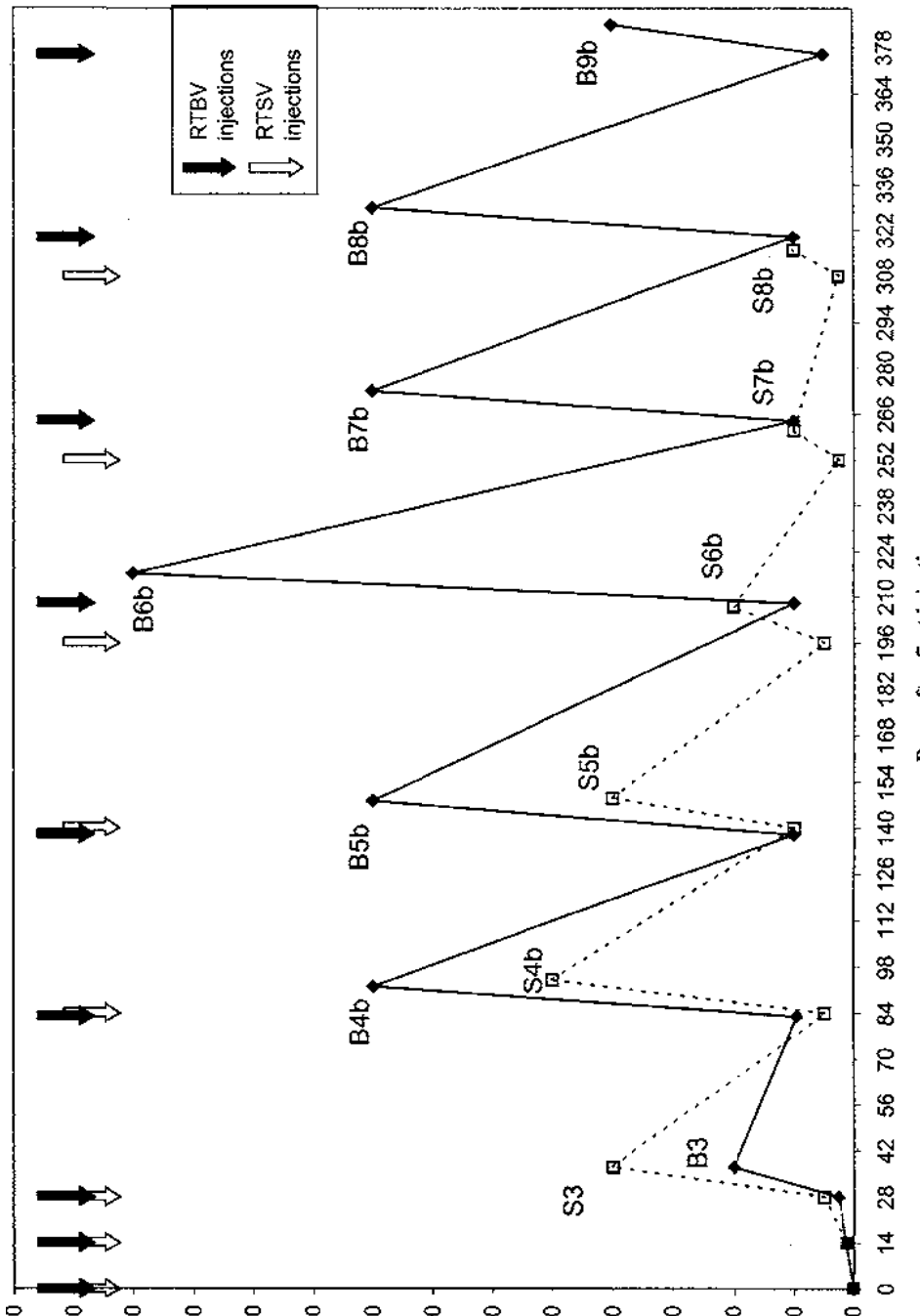

FIG. 1. Antibody titres determined by the ring-interface assay in serum from two rabbits following injections with purified RTBV and RTSV.

greenhouses of the International Rice Research Institute. Virions were purified from host tissues following the procedures of Cabauatan and Hibino (1988).

For antisera production the rabbits were first given a 600 µl subcutaneous injection containing 0.2 mg virions in 0.01 M-phosphate buffer, pH 7.4, and Freund's complete adjuvant (1:1). This was followed by two similar doses at 2 and 4 weeks, each containing Freund's incomplete adjuvant. Test bleeds (1–2 ml) were taken before each injection. The first main bleeds, of 20–30 ml, were collected 9 days after the third injections. The rabbits were allowed to rest for 6 weeks after the third injection, and were then given booster injections with 0.2 mg antigen plus Freund's incomplete adjuvant (1:1). Ten days later the rabbits were bled (30 ml) again. This cycle of approximately six weeks rest, then injecting with 0.2 mg antigen plus Freund's incomplete adjuvant (1:1) and bleeding after nine or 10 days was continued up to the ninth antigen injection (Figure 1).

The titre of each of the batches of antisera for both RTBV and RTSV was tested by the ring-interface precipitin test (Van Regenmortel, 1982) against purified virus (Figure 1). To establish DAS-ELISAs, immunoglobulins (primarily IgG) were purified by ammonium sulfate precipitation and diethylaminoethyl (DEAE)-cellulose chromatography (Harlow & Lane, 1988). IgGs were adjusted to a final concentration of 1 mg ml^{-1} (A_{280} = 1.35). Purified IgGs from the respective bleeds were designated as IgGB3–IgGB9 (anti RTBV) and IgGS3–IgGS9 (anti RTSV). Antibody-alkaline phosphate conjugates were prepared with gluteraldehyde following the procedure of Clark and Adams (1977).

Characterization of Antisera

Using RTBV–, RTSV– and RTBV + RTSV-infected and uninoculated leaf samples from the glasshouse, the sensitivity and specificity of each antiserum was tested by DAS-ELISA, and the better batches were also tested by tissue-printing. First, a 10-fold dilution series of each batch of IgG from 10^{-3} to 10^{-6} in coating buffer (0.05 M-sodium carbonate, pH 9.6) was tested for trapping in DAS-ELISA with sap diluted 10^{-1} and antibody-alkaline phosphatase conjugate diluted 10^{-3}. Then each was tested with the trapping antibody and conjugate dilutions being fixed at 10^{-1} while the plant sap was diluted 1:10, 1:20 and 1:40 (w:v) in phosphate-buffered saline (PBS).

The antisera were also tested by DAS-ELISA for their reaction against 10 field isolates (combined RTSV and RTBV) that were collected from North Cotobato, Philippines, and maintained in cultivar TNI in the greenhouse at IRRI. These isolates differed in symptom severity in TNI, and in virulence on six differential rice varieties unpublished data.

Tissue Printing and Antiserum Cross-Adsorption

Initially, nitrocellulose (Bio-Rad transblot 0.45 µm) membrane was used for tissue-printing. Fresh stems of rice plants (tungro infected and healthy) were cut (transverse or oblique) with a new, clean disposable razor blade, and the cut surfaces immediately 'printed' onto the membrane. After allowing the prints to dry for about 10 min, the remaining free binding sites on the membranes were blocked by immersing them in 5% (w/v) skimmed milk powder (Carnation) in PBS for 30 min. Membranes were then probed either with crude antiserum, purified IgG or IgG-alkaline phosphatase conjugate diluted in skimmed milk blocking solution. Antiserum dilutions of 1:250 to 1:2000 were tested, with the ratio of volume of solution to area of membrane kept at 160 µl cm^{-2}. When crude antiserum or non-conjugated IgGs were used, the membranes were washed with PBS and then probed with a goat-anti-rabbit IgG-alkaline phosphatase conjugate (Sigma A3687) diluted 1:10 000 (v:v) in skimmed milk blocking solution. After a final wash with PBS, the membranes were developed with BCIP/NBT liquid substrate (Sigma B1911). Subsequently, polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore) and shortened probing/development procedures were tested.

In order to reduce background signal on tissue-print membranes from non-specific antibody binding to healthy plant components on the membranes, direct cross-adsorption was used, initially. Healthy rice plant homogenate (either whole, or supernatant after centrifugation) was added to dilute antiserum (final dilution 10 mg rice plant per 1 ml of dilute antiserum), and the mixture incubated at room temperature for one hour before using it to probe tissue-print membranes. Subsequently, the healthy plant components were immobilised on a solid support. For small quantities of antiserum, fresh pieces of nitrocellulose membrane (2×4 cm) were saturated with healthy rice plant homogenate (1:4, w:v, in distilled water) for 5 min. Any unreacted sites were blocked with skimmed milk blocking solution, and then these membranes were incubated in diluted antiserum (1 cm^2 ml^{-1} for 1 h at room temperature prior to using the diluted antiserum for probing tissue-print membranes. To remove healthy plant-reacting antibodies from larger quantities of antiserum, healthy-plant component-Sepharose affinity chromatography columns were used. To create these columns, cyanogen bromide activated Sepharose (CNBr-activated Sepharose Fast-flow-4; Pharmacia Biotech) was treated with 1 M-HCl according to manufacturers instructions (1 g dry Sepharose yields approximately 3 ml of matrix). Then, 3 ml prepared Sepharose was incubated with 10 ml of healthy rice homogenate (2 g healthy rice plant tissue homogenised in 10 ml coupling buffer (100 mM-$NaHCO_3$, 500 mM-NaCl, pH 8.3) for 4 h at room temperature with gentle shaking. The Sepharose was then washed with 200 mM-glycine in coupling buffer for 30 min to block any un-reacted sites. The Sepharose (which by this time was a pale dirty-yellow colour) was then loaded into glass-wool-plugged plastic syringe barrels (1.5 ml Sepharose in a 3 ml syringe barrel) to form small affinity columns with a bed volume of approximately 1 ml. The columns were washed with about 100 ml PBS. Then, 1 ml of purified IgG was loaded per column, and incubated at room temperature for 4 h or overnight at 4 °C. The unbound, virus-specific IgGs were eluted with 2 ml PBS. The columns could be re-used by first removing the bound (plant component-cross-reacting) antibodies by washing with sodium acetate buffer (100 mM-sodium acetate adjusted to pH 4.0 with acetic acid) and then re-equilibrating with PBS.

RESULTS

Antisera Production

The antisera recovered following the first two injections gave very low ring-interface precipitin titers (dilution end point 1:20–1:80) for either virus, and hence were not considered further (Figure 1). For RTBV, a maximum dilution end-point (DEP) of 1:1900 was obtained for antiserum batch B6b, 31 weeks after first injection. Afterwards, the DEP declined reaching a level of 1:960 for batch B9b at 55 weeks after first injection. For RTSV antisera, the trend was similar, though the maximum DEP of 1:800 was recorded for batch S4b at 94 days after first injection. Subsequent injections failed to elicit such a strong response (Figure 1).

Antisera Characterization

Based on the ring-interface test results, all seven batches of RTBV and RTSV antisera were selected for further testing. For each batch, IgG was purified, conjugated with alkaline phosphatase, and a DAS-ELISA developed. For RTBV, clear differences in the titre of the seven batches were observed at trapping-IgG dilution 10^{-3}. At that dilution, batches IgGB4b and IgGB6b gave the highest A_{405} values in their reaction with RTBV-infected plant extracts, followed by batches IgGB3 and IgGB5b (Figure 2(a)). All other batches showed A_{405} values ≤ 0.5. At increased dilutions of the IgGs, the same trend was observed although A_{405} values were lower. For RTSV, differences among the batches were also clearest at the trapping-IgG dilution of 10^{-3}. In this case, IgGS3 showed the greatest titre with RTSV-infected plant leaf samples followed by IgGS6b (Figure 2(b)). The lowest absorbency values were recorded

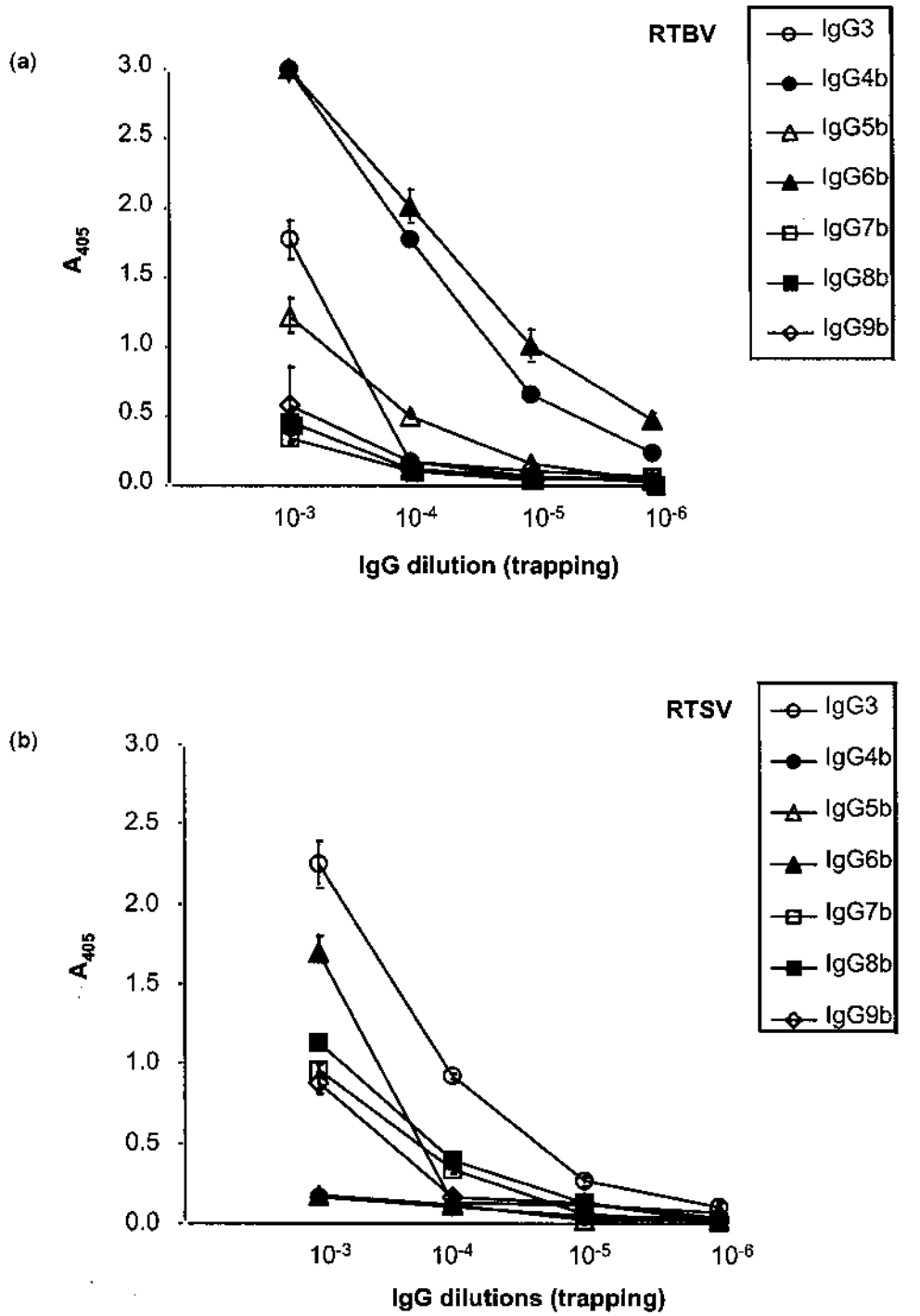

FIG. 2. Average absorbency values for 3 replicate reactions of either (a) rice tungro bacilliform virus (RTBV) or (b) rice tungro spherical virus (RTSV-) infected plant extracts when tested by ELISA against seven batches of their corresponding immunoglobulins IgG. Conjugate and sample dilutions were maintained at 10^{-3} and 10^{-1}, respectively. Absorbency values were corrected for the background readings from healthy plant extracts. Bars = standard errors.

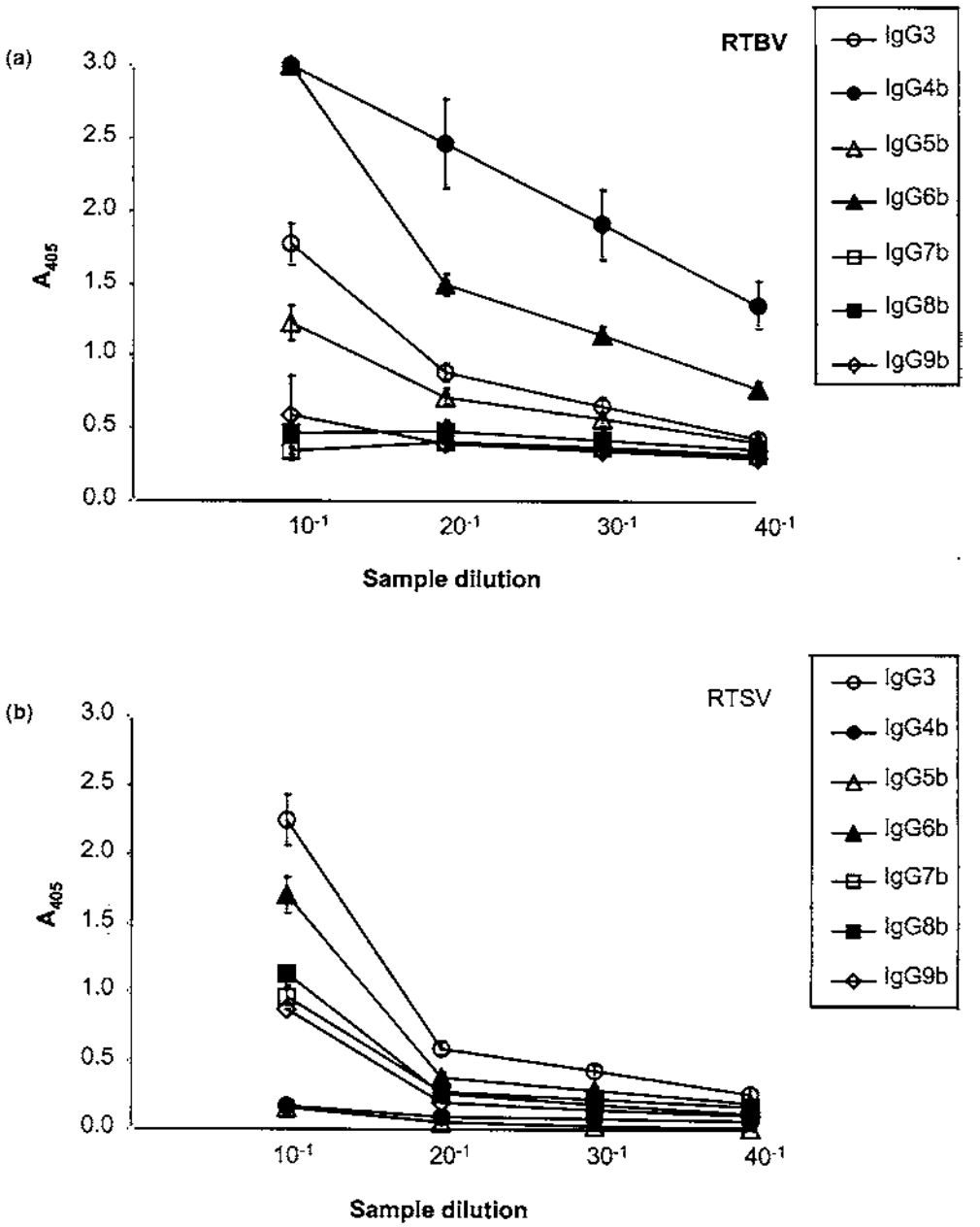

FIG. 3. Average absorbency values for three replicate reactions of either (a) rice tungro bacilliform virus (RTBV-) or (b) rice tungro spherical virus (RTSV-) infected plant extracts when tested by ELISA against seven batches of their corresponding immunoglobulins (IgG). Trapping antibody and conjugate were maintained at 10^{-3} dilution. Absorbency values were corrected for the background readings from healthy plant extracts. Bars = standard errors.

TABLE 1. Reactions of the different antisera batches in DAS-ELISA against a range of different tungro disease (RTBV + RTSV) isolates from North Cotobato, Philippines, maintained in c.v. TN1 in the glasshouse at IRRI. Trapping antibody dilution was 10^{-3}, sample dilution was 10^{-1}, and antibody-alkaline phosphatase dilution was 10^{-3}. Means followed by a common letter are not significantly different at the 5% level by Duncan's multiple range test

(a) A_{405} values (mean of two reactions) for RTBV antibodies in DAS-ELISA

Isolate	IgGB3	IgGB4	IgGB5	IgGB6	IgGB7	IgGB8	IgGB9
i1	0.96	2.70	0.82	2.28	0.35	0.33	0.28
i2	0.76	2.91	0.82	2.85	0.37	0.37	0.41
i3	0.99	2.56	0.95	2.13	0.40	0.31	0.25
i4	0.79	2.44	7.40	2.40	0.24	0.38	0.30
i6	0.66	2.51	0.85	2.41	0.28	0.30	0.30
i8	1.02	2.35	0.93	2.45	0.51	0.46	0.50
i9	0.74	2.59	0.85	2.68	0.31	0.32	0.36
i10	0.86	2.39	0.90	2.23	0.35	0.47	0.36
i11	0.66	2.12	0.64	2.32	0.36	0.35	0.39
i13	0.65	2.12	0.67	2.38	0.48	0.50	0.46
RTBV[a]	0.83	2.50	0.93	2.71	0.80	0.81	0.81
RTBV + RTSV[b]	1.14	3.00	1.26	3.00	0.87	0.97	0.90
	0.84[b]	2.52[a]	1.42[b]	2.49[a]	0.44[c]	0.46[c]	0.44[c]

(b) A_{405} values (mean of two reactions) for RTSV antibodies in DAS-ELISA

Isolate	IgGS3	IgGS4b	IgGS5b	IgGS6b	IgGS7b	IgGS8b	IgGS9b
i1	2.57	1.67	2.17	2.72	1.84	1.80	1.62
i2	2.36	1.34	1.77	2.65	1.53	1.50	1.53
i3	2.57	1.65	2.11	2.67	1.81	1.83	1.47
i4	1.93	1.30	1.51	2.17	1.25	1.26	1.38
i6	2.53	1.54	1.96	2.54	1.74	1.69	1.49
i8	2.80	1.84	2.40	2.73	2.02	2.26	2.29
i9	2.54	1.52	2.01	2.71	1.84	1.69	1.90
i10	2.50	1.51	2.05	2.51	1.83	1.93	1.55
i11	2.15	1.20	1.68	2.32	1.63	1.67	1.35
i13	2.47	1.23	1.88	2.20	1.62	1.91	1.03
RTSV[a]	2.66	1.29	1.94	2.60	1.76	2.37	1.76
RTBV + RTSV[b]	3.00	1.73	2.75	3.00	2.56	2.66	1.72
Mean	2.51[a]	1.49[d]	2.02[b]	2.57[a]	1.79[bc]	1.88[bc]	1.59[cd]

[a] Standard lab isolate of RTBV or RTSV alone.
[b] Standard lab isolates of RTBV and RTSV in the same plant.

from batches IgGS4b and IgGS5b. Only IgGS3 results were consistent at dilution 10^{-4} but could not be seen in the increased dilutions (Figure 2).

Since differences among the batches were clearest at IgG dilution 10^{-3}, this dilution was then used against a series of sample dilutions 1:10 to 1:40, to examine the sensitivity of detection of the various batches of IgG for both RTBV and RTSV. For RTBV, at sample dilution 1:10, both IgGB4b and IgGB6b gave A_{405} readings off the scale in reaction with RTBV-infected plant extracts. This result was consistent with that obtained from the previous experiment. However, at increased sample dilutions, IgGB4b showed significantly greater A_{405} values than IgGB6b. The DAS-ELISA was subsequently optimised for these two antiserum batches to have a trapping-antibody dilution of 1:2000, a sample dilution of 1:20

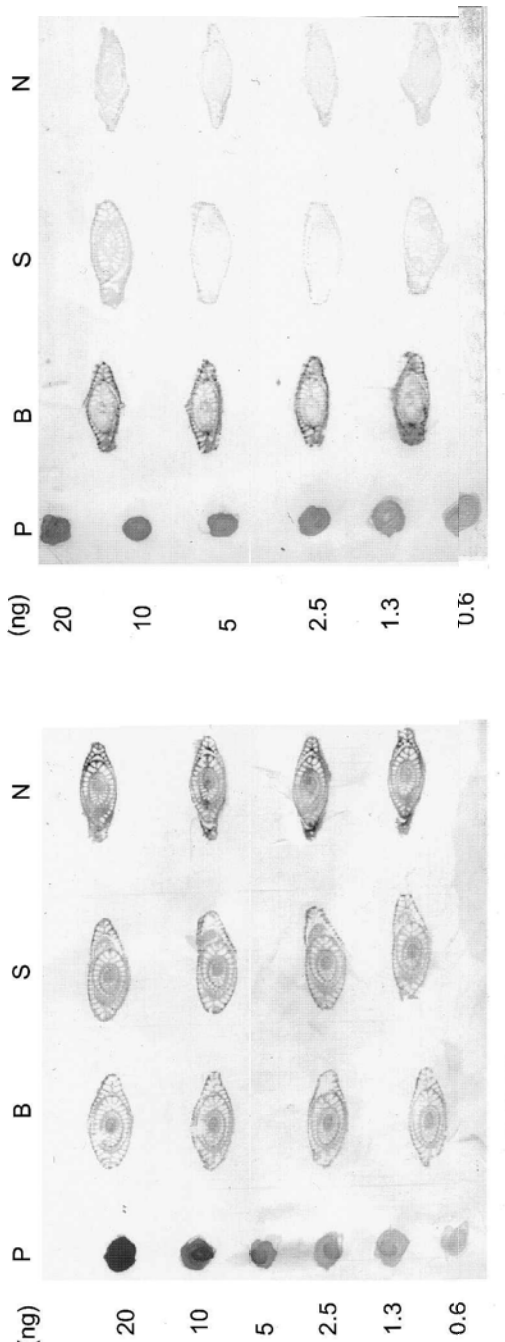

FIG. 4. Tissue prints from RTBV-infected plants (B), RTSV-infected plants (S), and healthy (ELISA-negative) plants (N) when hybridized with (a) complete IgGB6b and (b) IgGB6b cross-absorbed with healthy plant components in the sepharose column. IgGs were used at 10^{-3} dilution and goat-anti-rabbit-alkaline phosphatase conjugate at dilution 10^{-4}. Columns of dots at the left edge of each membrane (P) are a dilution series of purified virus; numbers to the left are approximate weights of purified virus in each dot.

and an antibody-conjugate dilution of 10^{-3} (data not shown). IgGB3 and IgGB5b gave A_{405} > 1.0 with a trapping-antibody dilution of 10^{-3} and a RTBV-infected sample dilution of 1:10 (Figure 3(a)). For RTSV, the affinities of the different antiserum batches could be differentiated only at sample dilution 1:10. IgGS3 gave the highest A_{405} value at this dilution (Figure 3(b)).

No differences in strain specificity could be detected among the antisera when they were tested in DAS-ELISA against the 11 field isolates of tungro. Here, as in the tests with the standard lab isolates, IgGB4b and IgGB6b, and IgGS3 and IgGS6b had broad spectrum detection capacities and were significantly better than the other IgG batches for detecting the different isolates of tungro (Table 1).

Tissue Printing and Antiserum Cross-adsorption

Based on the DAS-ELISA results, IgGB4b, IgGB6b, IgGS3 and IgGS6b were selected and tested for their sensitivity in, and applicability to, tissue printing. Initial results showed a persistence of a high 'healthy' background with all the four antisera (data not shown). This background was reduced when the nitrocellulose membrane was replaced with PVDF membrane. When the IgGs were cross-adsorbed with healthy rice plant components either by direct cross-adsorption to soluble components or by passage through the prepared CNBr column, IgGB6b used at 1:2000 dilution showed a highly specific reaction with RTBV-infected plant prints (Figure 4). Results for RTSV were inconsistent, and antisera cross-adsorbed with healthy plant components produced only very weak signals in tissue printing.

When a standard lab batch of antiserum (produced using four intramuscular injections followed by one inter-veinal injection of the rabbit at weekly and bi-weekly intervals, respectively (Cabauatan & Hibino, 1988)) was compared with IgGB6b in tissue printing, it was found to have less cross-reaction to healthy plant components. However, following cross-adsorption, it had to be used at a lesser dilution (between 1:500 and 1:1000 compared to 1:2000 for IgGB6b).

DISCUSSION

The repeated injection procedure used in this study worked well for RTBV, producing the classic weak primary and repeated stronger secondary responses (Figure 1). The antiserum produced 10 days after the sixth injection had the greatest affinity for RTBV particles in the ring-interface test. The lower titres measured in the other batches may be because the sixth injection was not given until 70 days after the fifth, whereas the other injections (except the first three) were all given at about 50 days after the previous. Thus, the titre of anti-virus antibodies in the rabbit at these other injection times may still have been great, causing rapid elimination of the antigen, and hence not such a strong response.

By the ring-interface assay, the primary response of the rabbit injected with RTSV appears to be stronger than that observed for RTBV, but the secondary responses are weaker. The relatively low titres for all the anti-RTSV batches may be due to the virus being less antigenic, or inherent differences in the rabbits used (the latter highlights one of the problems of using only one rabbit per virus). Also, as with the RTBV injections, it may be that insufficient time was left between injections to allow the titre of specific circulating antibodies to drop sufficiently for them not to eliminate the majority of the antigen before that antigen had had time to elicit a secondary response.

The relative affinities of the different batches of antisera appeared to differ depending on how they were measured (ring-interface or DAS-ELISA). The ring-interface test is not very precise since it does not measure over a continuous scale, but rather relies on the assessor being able to see the precipitation at different dilutions of the antiserum. Also, the ring-interface test also does not discriminate between immunoglobulin types, whereas for the

ELISA the antisera were purified by ammonium sulfate precipitation and DEAE chromatography, which favours IgGs. It is well documented that secondary immune responses tend to have a higher proportion of IgG compared with the primary response, and that the IgGs in the early secondary responses tend to have lower avidity (higher specificity) than those produced later (Gibbs & Harrison, 1976).

The affinities of the different batches of antiserum differed dramatically when tested as the trapping antibodies in DAS-ELISA. At a trapping dilution of 10^{-3} (equals approximately 100 ng serum proteins/well), the system was saturated (i.e. $A_{405} > 2.999$ with 1:10 diluted sap) for IgGB4b and IgGB6b with RTBV infected plant extracts. For all the other RTBV batches and the RTSV batches, at 10^{-3} trapping dilution, it was the titre of the antibody that was the limiting factor (Figure 2). The other antisera used routinely in the lab (e.g. Cabauatan & Hibino, 1988) under the same assay conditions have been found to be optimum at trapping antibody dilutions of between 1:500 and 1:700. This relates to a 1:10 (w:v) dilution of sap from a heavily infected plant giving an A_{405} (1 h) reading of about 2. Thus, by this test, only IgGB4b and IgGB6b performed better than the other lab antisera.

The titre and specificity of polyclonal antiserum is largely dependent on the immunisation protocol followed. The protocol used in this study varied slightly from that used by Cabauatan and Hibino (1988). In their study, the production of RTBV and RTSV antisera was done using four intramuscular injections followed by one inter-veinal injection at weekly and bi-weekly intervals, respectively, with purified RTBV and RTSV suspensions. The results presented here suggest that the slightly less risky procedure of multiple subcutaneous injections can produce antisera with similar or slightly better qualities. Two of the anti-RTBV batches produced had significantly greater titre than polyclonal antisera produced by other injection protocols. More recently, other workers have been looking into alternative methods for immunizing rabbits. Lima *et al.* (1998) found that simple oral immunizations with clarified infected plant sap gave reasonable quality antisera against cowpea mosaic comovirus and papaya lethal yellowing virus.

It is possible that had it been possible to produce monoclonal antibodies to these viruses, that these would have had greater titre and be more specific than the pAbs that were produced. However, facilities for the reliable and economic production of monoclonal antibodies were not available locally, and there would have been the risk that any monoclonal antibodies produced would have been too specific and would not have reacted with the wide range of tungro isolates that the polyclonal antisera reacted to.

Another approach being more widely adopted for the production of high titre (low avidity) anti-plant-virus antisera is the use of recombinant viral coat proteins expressed in *E. coli* as antigen for pAb production. Relatively large amounts of these expressed proteins can be produced, and since they have not been in contact with host plants, the resulting antisera are less likely to be contaminated with antibodies with cross-reactivity to healthy plant components. This approach requires the use of considerable resources that were not available locally. However, separate polyclonal antisera to the three coat protein components of RTSV and the single component of RTBV have been made by this procedure (R. Hull, John Innes Centre, personal communication). It would be interesting to compare the specificity and sensitivity of these antibodies against recombinant proteins with the ones produced in this study.

Serological assays can be a rapid way to assess whether a cultivar is resistant to a virus, though they can create confusion when plants demonstrate tolerance. Because inoculation with RTBV and or RTSV relies on the vector, it is difficult to inoculate reliably large numbers of plants under controlled conditions, and escapes are common. Thus, to make the work of the rice breeders more efficient, it is important to identify or develop methods which are suitably reliable and accurate for detecting the rice tungro pathogens. ELISAs based on the best batches of antisera produced here were sensitive and as reliable as those used routinely in the lab and based on other antisera. However, the 96-well format of ELISA is not practical in many situations where resources are poor, so the antisera were tested in an alternative

tissue-print assay. This worked well with two batches of the anti-RTBV antisera when these were first cross-adsorbed with healthy plant components and PVDF membrane was used. Nitrocellulose has a higher protein-binding capacity than PVDF (Millipore 1990), and thus was probably holding more healthy plant components than the PVDF membrane, resulting in a higher 'healthy background' when nitrocellulose was used.

Although some of the batches of anti-RTSV antisera worked well in DAS-ELIDA, disappointingly, none could not be made to work the tissue-printing system. On tissue-print membranes (either nitrocellulose or PVDF) the RTSV antisera produced very high "healthy background" when used directly, while cross-adsorption resulted in weak signal with all samples on PVDF membrane. One distinct advantage of the tissue-printing procedure over traditional 96-well plate ELISA is that it requires very little sample preparation since sap extraction by grinding or pressing, which is very difficult to do consistently with rice, is dispensed with. However, the difference between the signals obtained with RTBV and RTSV in tissue printing may reflect the observation that RTSV is found only in the phloem cells, while RTBV particles have been observed in both phloem and xylem (Sta. Cruz *et al.*, 1993). Thus, although grinding will release both types of virus particle, it may be that the RTBV particles are released more readily onto the membrane from the cut surface. Also, RTSV particles may be more tightly bound to the phloem cell walls than RTBV, or they may have different charge characteristics and so do not bind to the PVDF membrane so tightly.

Since, in the field, vector transmission of RTBV is reliant on a transmission factor from RTSV, it is likely that the majority of plants detected as having RTBV in the field will also be infected with RTSV. Thus, despite not being able to detect RTSV by the tissue-printing method, the test for RTBV should prove useful to rice breeders and extension services in Asia. Because the tissue-printing procedure is relatively simple, uses little antiserum and does not require other equipment, the cost of each reaction is comparatively low. A 4.5 × 7.5 cm membrane was a manageable size to use in tissue printing, and could hold up to 40 stem or petiole prints (= 20 samples if duplicate prints are made from each sample). A number of kits based on this procedure with these antisera are currently being evaluated in India, Indonesia and the Philippines.

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